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ANALYZING GENETIC DIVERSITY IN CONIFERS...
isozyme resolution by starch gel electrophoresis

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Abstract: Enzymes in forest tree materials can be resolved by starch gel electrophoresis. A gel slab is prepared in a mold assembled from glass and plastic. Wicks containing an aqueous extract of macerated plant material are inserted in the gel and processed. The gel is sliced, stained, examined, and photographed. Isozyme bands produced by differential migration of enzymes indicate genetic segregation and recombination. This technique is familiar in other uses, but the procedure described here is specifically adapted to conifers.

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The resolution of enzymes from the aqueous extract of macerated plant material is a precise tool with wide application in the genetic analysis of forest trees. In this procedure, proteins separated by electrophoresis are stained to reveal specific classes of enzymes. Genetic diversity is identified when the stained isozyme bands show migration at different rates. Interpretation of the isozyme patterns reveals segregation and recombination in families according to simple expectations of Mendelian inheritance.

Genetic theory holds that nuclear genes contain molecular codes responsible for the precise sequence of amino acids in specific proteins. Slight differences between nuclear codes bring about altered sequences within the protein, and in some instances, a resulting change in the net electrostatic charge on the protein. Thus, in a diploid organism, the products from different loci and allelic products from the same loci can carry charge differences. During electrophoresis a direct electric current is applied to gel medium containing plant extracts. The current, in conjunction with the protein's net charge, produces differential electrophoretic mobility.

Electrophoresis is an old and well-known process for protein separation. Of two standard techniques—disc and gel electrophoresis—the gel technique is most practical for genetic studies of forest trees. The essentials of the technique were described by Smithies in 1955.[1] It consists of casting the support medium in the form of a slab. Paper wicks containing sample extracts are inserted into the slab and a current applied. Gels can be formulated from several materials, but starch and acrylamide are most commonly used.

Proteins in pine pollen were investigated through electrophoresis as early as 1964.[2] Later, soluble proteins from female gametophytes and embryos of two pine species and a spruce were resolved.[3] In 1966, population geneticists became aware of the

potential of enzyme separation techniques,[4] and demonstrated their applicability to analysis of natural populations.[5] Since then, enzyme inheritance has been described in numerous organisms. Researchers in Professor R. W. Allard's laboratory at the University of California, Davis, have developed and adapted procedures which give unequivocal separations of isozyme bands and allow for the genetic assay of large numbers of individuals.[6]

This note describes some procedures proven to be applicable to pines[7] so that other geneticists working with conifers can bypass trivial problems of technique.

STARCH GEL TECHNIQUE

Good references for starch gel procedure include those by Smithies,[1] Shaw and Koen[8] and Brewbaker *et al.*[9] Scandalios[10] described a formulation for both gel preparations and enzyme stains. These formulations are appropriate for conifer enzyme separations. Kristjansson[11] relates in essential detail the procedures followed at the University of California, Davis, which are appropriate for pine.

Gel Preparation

Fresh gels should be prepared on the day of an electrophoresis run. For 12 percent gels, add 84 g. of hydrolyzed starch to 700 ml. of gel buffer solution (630 ml. tris-citric buffer, pH 8.3, 0.2 M, and 70 ml. lithium-borate buffer, pH 8.3, 0.2 M).[10] Heat 500 ml. of the buffer to a boil and add it rapidly to the starch, which has been suspended in the remaining 200 ml. of buffer. During addition and for a short period thereafter, swirl the flask vigorously 60 times or for 20 seconds. Immediately degas the viscous mass by applying a vacuum, slowly at first, as the gel tends to fill completely with air bubbles. Allow degassing to continue for about 1 minute. Disconnect the vacuum and pour medium directly into the prepared gel molds. The quantity of medium fills two molds.

Molds should be assembled before the gel is prepared. They consist of a plate glass bottom, plastic spacer held in place with paper clips, and a plate glass top *(fig. 1)*. Both glass plates are 16.5 cm. by 25 cm. Attach clips to the bottom plate, two clips per edge. The spacers are 9 mm. thick and 1.2 cm. wide; the long ones are 21.5 cm., and those on each end are 16.5 cm., the same dimension as the glass plate. Enough medium is poured in each mold to reach the inner margins of the spacers. Bring the top plate down on the gel, starting from one end and progressing slowly in much the same manner that a cover slip is placed on a microscope slide. For the best consistency, cool the gels at room temperature for 2 to 3 hours. They can be cooled more rapidly by refrigeration.

Preparation of Plant Material

The presence or absence of specific enzyme bands in the processed gel depends on both the physiological development of the plant and the plant part that is sampled.[10,12] Procedures will be described here for studying enzymes from conifers at germination, but the technique can be extended to various other materials.

Seeds of hard pines are stratified 30 days on moist filter paper at 3°C. and germinated at room temperature. An optimum period for alcohol dehydrogenase and leucine aminopeptidase analysis is the developmental stage in which the radicle of the embryo extends about 2 to 5 mm. beyond the seed coat. The embryo can be separated from the megagametophyte and both materials assayed. Place each material to be examined in a separate microsize disposable weigh tray *(fig. 2)*. For materials of small mass, add one drop of gel buffer solution. Then thoroughly macerate the plant material with a plastic rod. Pick up the aquaeous phase from the macerated fraction on paper wicks (0.4 by 1 cm.) cut from chromatography paper. Wicks should be thoroughly moistened but excess liquid should be blotted away. Place the prepared wicks in petri dishes and refrigerate them until all wicks are prepared.

Electrophoresis

Remove the top glass and clips from the prepared gel. Cut the gels once across their width at a distance of 5 cm. from one end. Separate the gel at the cut and insert the prepared wicks *(fig. 3)*. Wicks can be inserted with as little as 1 mm. between them; 20 such wicks can be accommodated across a single gel. The wick size and spacing can be varied according to the needs of a particular experiment. Once wicks are inserted, place the end slice against the wicks, and cover the gels with wicks with plastic wrap.

To rig the gels with electrode connections *(fig. 4)*, place trays containing solutions (lithium-borate buffer, pH 8.3, 0.2 M) at either end of the gel. Place thin cellulose sponges saturated with electrode buffer in the buffer tray and lap them over each end of the gel. The plastic wrap should be turned back at each end and lapped under the sponge a short distance to give an even contact of sponge with gel surface. The wrap is lapped back over the top of the sponge and held in place with clips along the outer edge. Use platinum

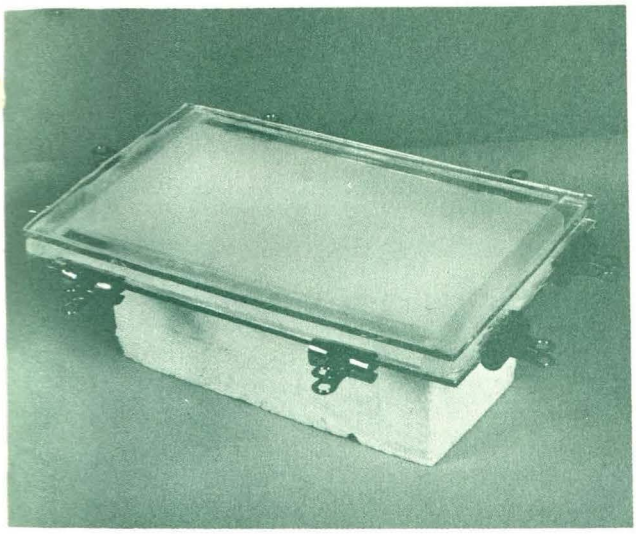

Figure 1—Mold with starch gel in place. Top and bottom pieces are of plate glass, separated by plastic spacers on all four sides. Clips hold the spacers in place. After the gel is poured, the top plate is laid over it.

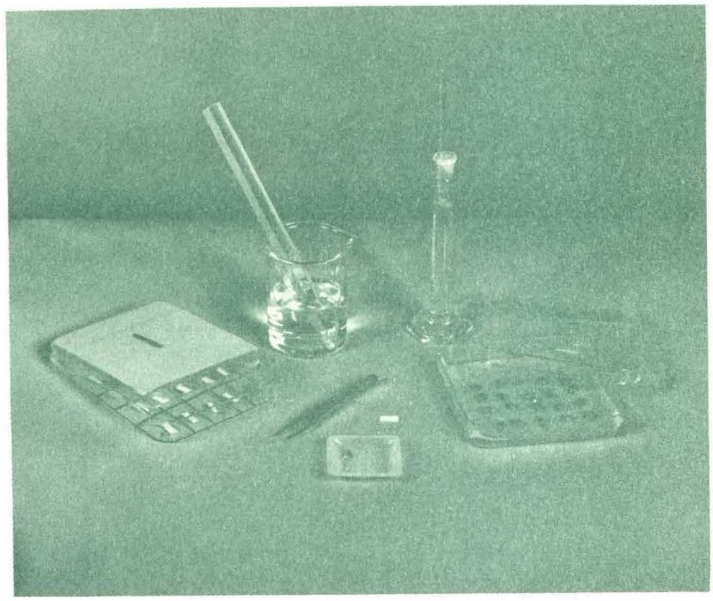

Figure 2—Materials for wick preparation. Paper wicks are saturated with the liquid fraction from plant material macerated with a plastic rod. Each material is prepared in a separate disposable weigh tray.

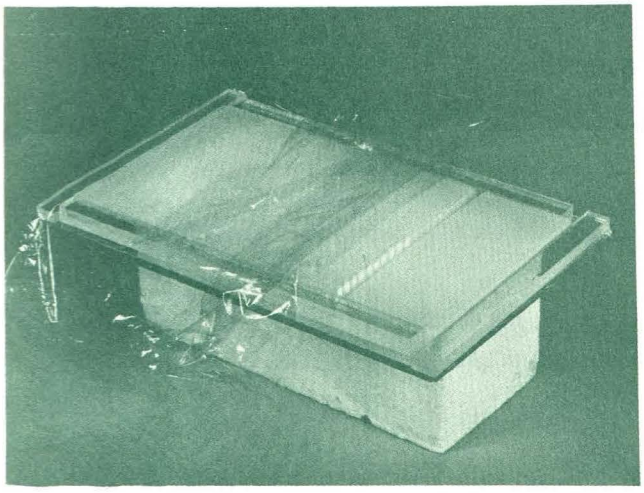

Figure 3—Placement of saturated wicks on cut gel surface. Gel, with wicks, is covered with plastic wrap.

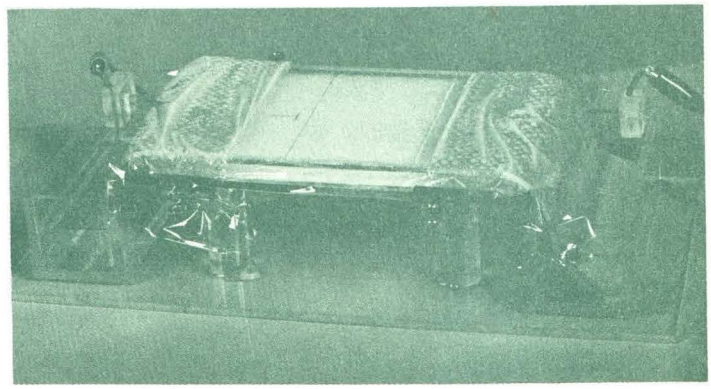

Figure 4—Gel electrode connections and solutions in place. Thin cellulose sponges lap over ends of gel and extend into trays of buffer solution. Platinum electrodes lead from the power source to the solution trays. The plastic wrap covers the gel and the sponges, and is clipped to the outer edge of the bottom plate.

electrodes to connect power sources and electrode tray solution.

Two precautions are taken to prevent heat build-up, which can result in uneven movement of fronts in the gel or denaturation of proteins. First, cover gels with a thin cellulose sponge and the sponge with crushed ice. Second, place the entire gel system in an electrically grounded refrigerator set to its coldest setting.

Various power sources have been used in this system, and all have given satisfactory results. I have used four power supplies, each delivering 400 v. d.c. with 100 ma. to process four gels. The negative pole from the power supply is connected to the electrode adjacent to the wick end of the gel slab. The positive pole is attached to the alternate electrode. Power supplies are located on top of the refrigerator and connecting wires to the electrodes bypass the door gasket. A small fan in the refrigerator, located near

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